AD_____

Award Number: DAMD17-99-2-9033

TITLE: In-Vitro Cytotoxicity Screening of Plant Extracts

PRINCIPAL INVESTIGATOR: Louis R. Barrows, Ph.D.

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, Utah 84102

REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (15 May 2002 - 14 May 2003)

4. TITLE AND SUBTITLE
In-Vitro Cytotoxicity Screening of Plant Extracts

5. FUNDING NUMBERS
DAMD17-99-2-9033

6. AUTHOR(S)
Louis R. Barrows, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Utah
Salt Lake City, Utah 84102

E-Mail: lbarrows@deans.pharm.utah.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
No Abstract Provided.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Annual Report for A.P. 6,
Louis R. Barrows

Foreword

The 2002-2003 year of activity in this portion of AP6 again had the novel anticancer activity of extracts SU 719 and SU 818 (grapefruit seed) as its main focus. The structure of the active component was determined to be, benzenemethanaminium, N,N-dimethyl-N-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]- (9Cl), commonly known as benzethonium chloride (BTC), was achieved this year with additional description of its biological activity. As discussed last year, topoisomerase II (top2) is the target of some of the most useful anti-cancer drugs. All of the clinically used top2 drugs act by stabilizing a drug-enzyme-DNA cleavable complex. BTC possesses a unique top2 activity *in vitro*. It induces human top2 to catenate plasmid DNA in a purified enzyme system, while at the same time only producing minimal DNA cleavage. This activity was confirmed this year in a second, filter-binding analysis of BTC-top2 interaction. We hypothesized that this catenation would correlate with the ability of BTC to aggregate DNA, but the iniital data are not clearly in support of this. BTC was tested for anti-cancer activity in animals bearing human tumors. At the doses tested BTC demonstrated powerful anti-cancer activity, but also potent toxicity in the treated animals. Further, full-scale animal experiments employing lower doses of BTC are justified. Additional work in '02-'03 included the screening of a limited set of extracts for anti-HIV activity and the identification of a particularly active $CHCl_2$ extract of the stem of *Jatropha curcas*.

Table of Contents

INTRODUCTION:

AP1 of the ICBG "Drug Development and Conservation of Biodiversity in West and Central Africa," provides data on the variety, distribution, abundance and dynamics of West and Central African flora. Other APs focus primarily on the chemistry and antiparasitic activities of traditionally used medicinal plants. The research conducted under AP 6 is concerned with development of African medicinal flora for their non-antiparasitic activities. Screening extracts from locally used plants can capitalize on traditional knowledge in order to provide information about the potential alternative uses of known plants. Some of Western medicine's most useful anti-cancer drugs have come from plants traditionally used for anti-parasitic or anti-helminthic activity. Screening of West African flora for anticancer activity can provide information on species utility not recognized by traditional healers because cancer is frequently not diagnosed at that level. Similarly, as discussed last year, many of the plants traditionally used to teat various illnesses also show important anti-viral activity, in particular, anti-HIV activity.

BODY:

As described in our approved statement of work, our approach is to prioritize cytotoxic extracts based on screens that can detect cancer relevant mechanisms of tumor cell killing. We first test extracts for the ability to kill human cancer cells in culture. We then utilize mammalian cell based screens to detect molecules in the extracts that interfere with DNA metabolism or the cell cycle. In the mechanism screens cytotoxic extracts/molecules are tested for enhanced cytotoxicity in mutant cell lines that lack various cancer predisposition/tumor suppressor gene functions (caretaker functions). Cytotoxic extracts/molecules showing no DNA directed activity are tested by flow cytometry for the ability to disturb progression of normal cells through the cell cycle (tumor suppressor gatekeeper functions). These assays were described in previous reports. Follow up on this work is presented here.

BTC The principle focus of work this year was the grapefruit seed extracts SU 719 and SU 818. Work reported last year demonstrated a topoisomerase II (top2) mechanism of toxicity for the active component of these extracts. Top2 is the target of some of the most useful anti-cancer drugs. All of the clinically used top2 drugs act by stabilizing a drug-enzyme-DNA cleavable complex. Here we report the structure of the active component of 719, benzenemethanaminium, N,N-dimethyl-N-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]- (9Cl), commonly known as benzethonium chloride (BTC). Benzenemethanaminium, N,N-dimethyl-N-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]- (9Cl), is a cationic detergent. (Figure 1) This quaternary ammonium compound is used as a disinfectant and a cleansing agent. It was first reported as a component of grapefruit seed extract by von Woedtke et al. in 1999([1]).

---

[1] von Woedtke T, Schulter B, Pflegel P, Lindequist U, and Julich WD. Aspects of the antimicrobial efficacy of grapefruit seed extract and its preservative substances contained. *Pharmazie*. **1999**, 54(6): 454-6.

That study reported that commercial grapefruit seed extracts contained significant amounts of both BTC and methyl parabene and that the antimicrobial activity of the extracts was due to these compounds. This finding was confirmed and expanded upon by Takeoka et al in 2001([2]). We reported last year that BTC exerts a unique, top2 dependent, DNA catenation activity *in vitro*, and is preferentially toxic to top2 over-expressing yeast.

NMR experiments were performed at either 500 MHz or 125 MHz. Chemical shifts were calculated as parts per million shift from tetramethylsilane as referenced from residual non-deuterated solvent peaks. Structure conformation was obtained through comparison to the literature values (2) as well as through the use of 2-dimensional NMR experiments (COSY, gHSQC, HMBC). High-resolution mass spectrometry experiments confirmed the molecular formula. (+)HRFAB = 412.3217, $C_{27}H_{42}NO_2$req. 412.3215 (+0.2 mmu), additional info: CAS# 10172-60-8

Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Mass spectrometry was performed by Elliot M. Rachlin (Department of Chemistry). Dr. Rachlin performed all FAB experiments on a Finnigan Mat 95 High Resolution Gas Chromatograph/Mass Spectrometer with Finnigan MAT ICIS II operating system funded by NSF Grant CHE-9002690 and the University of Utah Institutional Funds Committee.

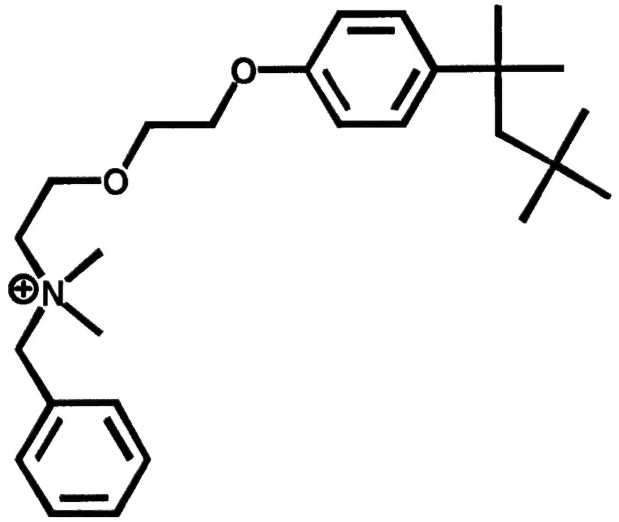

Figure 1 Benzenemethanaminium, N,N-dimethyl-N-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]- (9Cl).

---

[2] Takeoka G, Dao L, Wong RY, Lundin R, and Mahoney N. Identification of benzethonium chloride in commercial grapefruit seed extracts. *J. Agric. Food Chem.* **2001**, 49: 3316-3320.

BTC was tested for anti-cancer activity in a preliminary experiment using a dose range protocol. Approximately 250 mg of BTC was purified using the HPLC procedure developed and described in the 2001-2002 report. Nude mice were purchased from Charles Reiver Laboratories. Each was inoculated with 2 million KB cells on the shoulder. KB cells are a human epidermoid naso-pharyngeal cancer cell line that grows rapidly in mice and is responsive to top2 poisons. Treatment began when tumor volumes reached a minimum of 50 mg. Animal groups consisted of 3 control mice treated i.p. with treatment vehicle, and 4 mice treated with BTC administered i.p. in 0.1 ml 0.05% methylcarboxycellulose, 2% DMSO, in minimal essential medium. This was a dose range

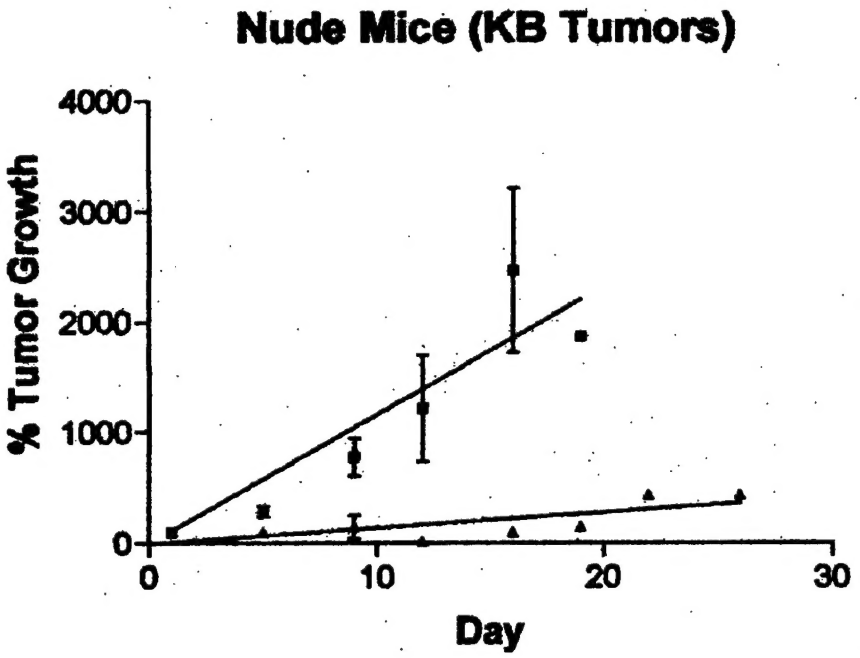

Figure 2 Tumor volume in control animals is indicated by the closed squares, tumor volume in BTC-treated animals is indicated by closed triangles.

experiment so each treated mouse received a unique BTC dose. Doses used were 20, 60, 70 and 80 mg/kg total (the 60 mg/kg mouse received 30 mg/kg on days 1 and 5 post staging). Dose range experiments are required by IACUC protocols to minimize the numbers of animals killed unnecessarily in anti-cancer studies. Tumor volumes were calculated following measurement of tumor size using an external caliper. Figure 2 shows the dramatic effect of BTC on tumor growth. However, the graph is misleading because only one mouse (the mouse receiving 80 mg/kg) survived beyond day 12. Surprisingly the two longest living treated mice were the ones that received the highest (70 and 80 mg/kg) doses. Thus, the tumor growth inhibition demonstrated in this experiment was not statistically significant, both because of the preliminary nature of this design, the dose

escalation protocol, and because of the small number of animals in the control and treatment groups. Nevertheless, the potent anticancer activity of BTC is evident. Significant work must be accomplished to establish dosing regimes that minimize the toxicity.

HIV Preliminary experience with the HIV screen has been achieved in our laboratories at the University of Utah. The protocol is briefly described here. To test extracts collected in Cameroon and Nigeria for anti-HIV activity, Dr. Barrows' laboratory has established the whole virus "CEM-TART" assay. In this system replication defective HIV-1 is grown in specially engineered human T-cells. Inhibition of virus production is monitored by trypan blue type colorimetric techniques. Such inhibition, at non-cytotoxic extract concentrations, may indicate potentially useful anti-HIV activity. The system to be employed here was developed by Chen and co-workers and optimized for screening by Kiser and colleagues ([3, 4]). Inhibition of virus production, and protection from toxicity indicates anti-HIV activity, thus the assay allows detection of anti-HIV components in complex mixtures. Also, because this is a whole virus production assay it is capable of detecting components active via a wide spectrum of anti-viral mechanisms.

"CEM-TART" and 1A2 cells are maintained in RPMI culture medium supplemented with 20% fetal bovine serum and standard concentrations of penicillin and streptomycin (10,000 U and ug/liter respectively). When grown at 37°C, 5% CO_2, and maintained at a concentration between 5 x 10^4/ml and 2 x 10^6/ml, the cells double approximately every 24 hours. For infection with virus, 2 x 10^6 "CEM-TART" cells are pelleted by centrifugation and resuspended in 0.5 ml of virus stock (freshly thawed). After 1 hr incubation at 37°C the cells are pelleted and resuspended at 2 x 10^5/ml for growth. Virus stock is prepared by centrifugation of a virus-producing culture of "CEM-TART" cells, 7 to 9 days after infection, and simply freezing aliquots of virus-containing supernatent. The viability of the "CEM-TART" cells does decrease somewhat with infection. To screen extracts for anti-HIV 1 activity, 1A2 cells will be infected as described above. Following overnight incubation, the cells are counted and dispensed into a 12 well microtiter culture dish at 20,000 cells/well in 1.5 ml culture medium. Serial dilutions of test extract are added to this (concentrations spanning 5 logs from 50 ug/ml to 0.05 ug/ml). Cultures are examined microscopically daily, and quantified every other day to generate cell growth inhibition curves. Cells are counted (by removing 10 ul of cells and mixing with an equal volume of trypan blue stain). Cytoprotection is visualized by plotting data using a custom designed program on a personal computer with Sigma Plot software.

We have received the CEM^{TART} ("CEM-TART"), 1A2 cell lines, the defective (MC99IIIB□Tat-Rev, also called vIIIB□$^{Tat/Rev}$) virus and protocols developed at the NIH from the AIDS Research and Reference Reagent Program. Data from the cytoprotection

---

[3] Chen H, Boyle TJ, Malim MH, Cullen BR, and Lyerly HK. Derivation of a biologically contained replication system for human immunodeficiency virus type 1. Proc Natl Acad Sci USA (1992) 89: 7678-7682.

[4] Kiser R, Makovsky S, Terpening SJ, Laing N, and Clanton DJ. Assessment of a cytoprotection for the discovery and evaluation of anti-human immunodeficiency virus

assay are plotted as 1A2 cell fractional survival up to 10 days after infection with HIV I tat⁻ rev⁻. Cultures are treated in quadruplicate with AZT as a positive control. Significant cytoprotection is evident at AZT concentrations of 0.5 to 5 ug/ml. Concentrations of AZT above 10 ug/ml are cytotoxic, resulting in a parabolic survival vs. dose curve. This modified assay uses trypan blue exclusion as an alternative means of quantifying "CEM-TART" and CEM-1A2 cell growth. The T-cell leukemia subline 1A2 is more sensitive than the TART line to cell killing by HIV infection and therefore it was selected as the primary cell line for this assay. When the total cell population was quantified daily over 11 days, it was found that the number of cells in non-infected and infected cultures decreased after approximately day 10. Figure 1 shows the protective effect of AZT on HIV infected 1A2 cells by following cell growth. The data shown are the numbers of live cells in the cultures, AZT was administered only on day 1. This is a very robust assay and yields an easily quantifiable signal when cultures are protected from HIV killing. It was also observed that syncitia formation serves as a robust measure of HIV infection in this system, and can be used as a qualitative assay of infection by observation under light microscopy. Drug synergy experiments will be performed by treating cells with combinations of drugs, as shown above for the cytoprotection assay. The DMSO concentration will be less than or equal to 1% culture volume. It will be a limitation of this work that only HIV-1M strain will be utilized, drugs that may be selective inhibitors of other sub-type cell fusion will not be identified.

Several extracts that have yielded statistically significant protection of HIV infected T-cells are the subjects of current investigation. The one shown here is a plant extract obtained through the current ICBG, "Drug Development and Conservation of Biodiversity in West and Central Africa," derivative FIRCA award "West African HIV Screening Capacity", Dr. Barrows PI. This funding supported the 2-month sabbatical of Dr. Kennedy Chah, from the InterCEDD, Nsukka, Nigeria, in Dr. Barrows' laboratory, 2003.

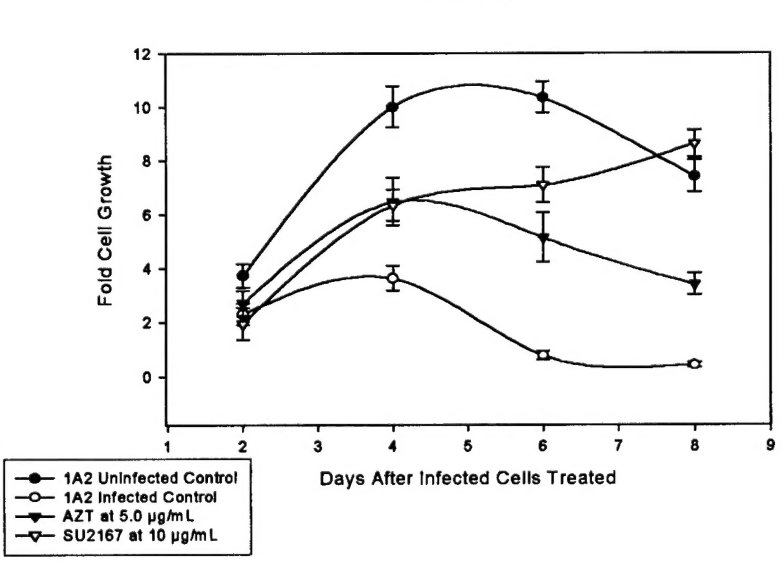

Figure 3

Anti-HIV activity of SU 2167 SU 2167 Is the $CHCl_2$ extract of the stem of *Jatropha curcas* (Euphorbiaceae), a plant used in Nigeria for the treatment of fevers. Significant protection of HIV infected T-cells has been obtained with this extract, with unusual effects on the kinetics of cell growth in infected cultures (Figure 3). This is a recent observation and fraction of the extract is just beginning. The observation shows our ability to assess extracts of plants for anti-HIV activity.

KEY RESEARCH ACCOMPLISHMENTS:

- Benzethonium chloride (BTC) was identified as responsible for the unique cytotoxic activity of extracts SU 719 and SU 818, its structure was identified using NMR and MS techniques.
- Approximately 250 mg of BTC was isolated at 99% purity.
- The anticancer activity of BTC was demonstrated in a xenograft experiment using nude mice and human KB tumors.
- The anti-HIV activity of SU 2167 was demonstrated using the whole HIV "TART" assay.
- Dr. Kennedy Chah trained in the TART assay and is sufficiently experienced in the technology to lead implementation of the assay at the InterCEDD.

REPORTABLE OUTCOMES:

- Benzethonium chloride (BTC) was identified as responsible for the unique cytotoxic activity of extracts SU 719 and SU 818, its structure was identified using NMR and MS techniques.
- The anticancer activity of BTC was demonstrated in a xenograft experiment using nude mice and human KB tumors.
- An AIDS Fogarty International Research Collaboration award, based on work described in previous years annual reports, has been funded and anti-HIV screening of African medicinal plant extracts began.
- The anti-HIV activity of SU 2167, the $CHCl_2$ extract of the stem of *Jatropha curcas* (Euphorbiaceae), was demonstrated using the whole HIV "TART" assay.
- Dr. Kennedy Chah trained in the TART assay and is sufficiently experienced in the technology to lead implementation of the assay at the InterCEDD.
- This research has supplied rotation projects and contributed to the training of three graduate (Ph.D.), and three undergraduate students at the University of Utah.

CONCLUSIONS:

Traditional medicines have been the source of several very important anticancer medicines (e.g., the vinca alkaloids, the podophyllotoxins and the camptothecins). The work in progress has focused on the traditionally used grapefruit seed extract as containing a constituent with significant anticancer potential. The structure of the active molecule was

been determined (benzethonium chloride, BTC) and significant progress has been made towards identification of its molecular mechanism. Additionally, BTC was purified in sufficient quantity to perform a preliminary tumor xenograft experiment. This experiment demonstrated potent anticancer activity, but also potent toxicity for BTC. Further, significant progress in establishment of the "TART" HIV assay for the discovery of anti-HIV extracts. This has provided training opportunities for collaborating scientists and identified extracts of African medicinal plants with significant anti-HIV activity, such as *Jatropha curcas*.